The Ulster Medical Journal, Volume 62, No. 1, pp. 21 – 28, April 1993.

Identification of carriers of haemophilia by polymerase chain reaction

P C Winter, Elizabeth E Butler

Accepted 18 December 1992.

SUMMARY

The gene for the coagulation protein factor VIII contains several common restriction fragment length polymorphisms which can be used to analyse the pattern of inheritance of factor VIII alleles within families. This can be exploited to identify carriers of haemophilia, an X-linked inherited disorder characterised by deficiency of factor VIII. In this study the polymerase chain reaction was used to analyse a polymorphism recognisd by the restriction enzyme BclI, located at intron 18 of the factor VIII gene. The restriction fragment patterns generated were used to track the inheritance of mutated factor VIII alleles within families allowing haemophilia carrier status to be determined in individuals at risk.

INTRODUCTION

Haemophilia is an X-linked inherited disorder which affects one male in 10,000. The condition is characterised by abnormal bleeding due to the deficiency of factor VIII, a protein necessary for normal coagulation. The deficiency is caused by heritable mutations within the factor VIII gene. Male off-spring of female carriers have a 50% chance of manifesting the condition. Female off-spring have a 50% chance of being carriers. Determination of haemophilia carrier status facilitates counselling of individuals at risk of having an affected child, allowing them to make informed choices regarding parenthood.

Initial attempts to identify carriers were based on measurements of the factor VIII coagulant activity in the plasma.[1] This approach proved unreliable due to the physiological variability in factor VIII levels and the random nature of X chromosome inactivaction in females.[2] The advent of recombinant DNA technology has permitted unequivocal identification of carriers in about two-thirds of cases using restriction fragment length polymorphisms (RFLPs).[3] These are naturally occurring variations in DNA sequence which alter the pattern of fragments produced when DNA corresponding to a particular gene is treated with a restriction endonuclease. The variations can be exploited to distinguish not only between genes carried by different individuals, but also between homologous pairs of genes (alleles) within individuals. Three common RFLPs occur in the factor VIII gene located at introns 18, 22, and 26. They are recognised by the

N. Ireland Regional Haemophilia Centre, Department of Haematology, Royal Group of Hospitals, Belfast BT12 6BA.

P C Winter, BSc, PhD, Clinical Scientist.

Elizabeth E Butler, MD, FRCPath, FRCP (Glasg), Consultant Haematologist.

restriction enzymes, *Bcl1*,[4] *Xbal*[5] and *Bgl1*.[6] By analysing the restriction fragment pattern of the factor VIII gene in DNA from a haemophilic patient using these enzymes, it is possible to identify the RFLP pattern associated with the X chromosome carrying the mutated gene. By comparing the pattern with that generated by the mother and sisters of the haemophilic, it is possible to trace the inheritance pattern of the mutated gene within the family and so to establish carrier members.

Restriction fragment length polymorphisms can be analysed using the polymerase chain reaction.[7] The technique allows an individual gene, contained within a complex mixture of genes such as chromosomal DNA, to be copied exclusively in a simple enzyme catalysed reaction until its DNA becomes the dominant species in the reaction mixture and enough is obtained to allow detailed analysis of that gene. Individual genes can be amplified about one million fold, and up to 1μg of specific DNA can be obtained.

This paper describes how the polymerase chain reaction was used to amplify the part of the factor VIII gene containing a RFLP recognised by the enzyme *Bcl1*, and how analysis of the restriction fragment pattern of the amplified DNA with *Bcl1* was used to identify carriers of haemophilia.

MATERIALS AND METHODS

Purification of chromosomal DNA: Human chromosomal DNA was prepared from 15ml of anti-coagulated blood.[8] A typical yield of 200μg of purified DNA was obtained.

Synthesis of oligonucleotide primers: Primers used to amplify the factor VIII intron 18 RFLP were synthesized by British Biotechnology Ltd. Their sequences have been described previously.[9]

Polymerase chain reaction (PCR): A DNA sequence corresponding to part of intron 18 of the factor VIII gene was amplified selectively from human chromosomal DNA. A reaction mixture (100μl) was prepared which contained: 1μg chromosomal DNA, 200μM each of dATP, dTTP, dGTP and dCTP (Pharmacia Ltd), 50 pmoles of each primer, 5 units of *Taq* DNA polymerase (Perkin Elmer Ltd) and PCR reaction buffer (10mM-Tris, pH 8·3, 50mM-KCl, 1·5mM-$Mgcl_2$ and 0·01% gelatin).

The chain reaction was carried out using an automated DNA thermal cycler (Perkin Elmer Ltd). Samples were initially denatured at 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 2 min. After the reaction was completed, samples were stored at 4°C pending analysis.

Digestion of amplified DNA by Bcl1 restriction enzyme: Amplified DNA was precipitated from the PCR reaction mixture with ethanol and redissolved in sterile water. It was digested with *Bcl1* according to the manufacturer's instructions (Promega Ltd).

Analysis of polymerase chain reaction products: DNA, amplified by PCR and digested by *Bcl1*, was analysed by electrophoresis on 7·5% polyacrylamide gels.[8] The gels were then stained with ethidium bromide and visualised under UV light.

Patients: A total of 139 haemophilia A patients from 89 families in Northern Ireland are registered with the Regional Haemophilia Centre, based in the department of haematology, Royal Victoria Hospital, Belfast. Only families of severely affected patients (59/139) are investigated routinely. Of ten families studied, haemophilia carrier status was successfully assigned in nine using the intron 18 RFLP in combination with RFLPs in introns 22 and 26 of the factor VIII gene and the extragenic X chromosome probe DX13.[10] The details of two families are described showing the detection of one carrier and two non-carriers.

RESULTS

Analysis of the polymerase chain reaction products by polyacrylamide gel electrophoresis revealed a single species of amplified DNA represented by a band whose length was calculated to be 142 base pairs by comparison with DNA marker molecules of known length. This value is in agreement with that predicted from the relative positions of the primers on the factor VIII gene.[9]

Analysis of amplified DNA which had been treated with *Bcl1* revealed several different banding patterns depending on the DNA sample used in the PCR reaction (Fig 1). In some cases, the banding pattern was unchanged from that observed for undigested samples and a single band of 142 base pairs was seen. This corresponded to samples in which the factor VIII intron 18 DNA sequence was such that no recognition site for *Bcl1* was present. In other cases, the 142 base pair band was lost and was replaced by two smaller bands of 99 and 43 base pairs. This corresponded to samples in which the DNA sequence was such that a *Bcl1* site was present resulting in the fragmentation of the 142 base pair band into two smaller bands on treatment with *Bcl1.* In some cases, treatment of amplified DNA with *Bcl1* produced a 142 base pair band as well as bands of 99 and 43

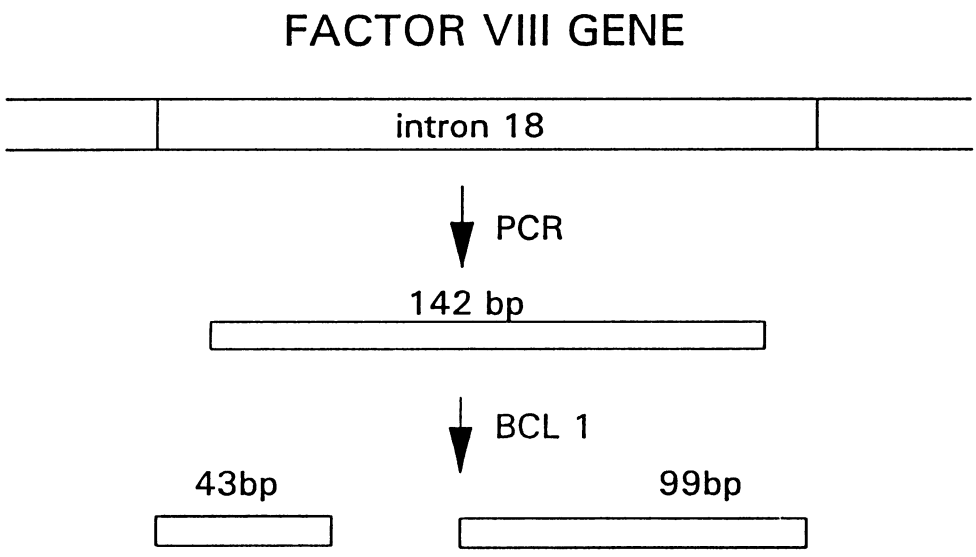

Fig 1. A 142 bp segment of intron 18 of the factor VIII gene containing a polymorphic *Bcl1* site was amplified by the polymerase chain reaction. Digestion of the reaction product with the enzyme *Bcl1* produced sub-fragments of 99 and 43 bp.

base pairs. This pattern represented heterozygous females with a *Bcl1* site present on one allele generating the 99/43 base pair bands and the other allele lacking a *Bcl1* site resulting in the 142 base pair band. It should be noted that the shortness of the 43 base pair band resulted in a reduced amount of ethidium bromide stain being bound causing it to be only faintly visible on gel photographs. The 99 base pair band is however clearly visible and indicates cleavage of the intron 18 *Bcl1* site.

TABLE

Intron 18 RFLP analysis in two families with haemophilia

Family	Individual	Genotype	Intron 18 *Bcl1* site	Phenotype
H1	I1	−/(99, 43)	+	unaffected
	I2	142 / (99, 43)	−/+	carrier
	II1	−/(99, 43)	+	haemophilic
	II2	142/−	−	unaffected
	II3	142 / (99, 43)	−/+	non-carrier
H2	I1	−/(99, 43)	+	unaffected
	I2	142 / (99, 43)	−/+	carrier
	II1	−/(99, 43)	+	haemophilic
	II2	142 / (99, 43)	−/+	non-carrier
	II3	(99, 43) / (99, 43)	+/+	carrier

Carriers of haemophilia were identified in several families by analysing the intron 18 RFLP in individuals. The strategy adopted was to identify the banding pattern associated with the X chromosome carried by a haemophilic and to use it to trace the pattern of inheritance of the affected X chromosome within the family. The results of typing for carrier status in families H1 and H2 are shown in the Table. The family tree and intron 18 PCR results for family H1 are shown in Fig 2. Interpretation of these PCR results is as follows: The father (I1) demonstrates two bands of 99 and 43 base pairs indicating that a *Bcl1* site is present on his single X chromosome at intron 18 of his factor VIII gene. The mother (I2) shows three bands of 142, 99 and 43 base pairs. This indicates that she is heterozygous with a *Bcl1* site present on one X chromosome (represented by the 99/43 base pair bands) but absent from the other (represented by the 142 base pair band). The son (II1) who suffers from haemophilia has the 99/43 base pair combination of bands indicating that of his mother's two X chromosomes, he has inherited the one which contains a *Bcl1* site and that this chromosome is associated with haemophilia in this family. In contrast, the unaffected brother (II2) has inherited the other maternal X chromosome indicated by the presence of a single band at 142 base pairs. The daughter's results (II3) indicate that she is heterozygous for the *Bcl1* site with all three bands present. The 99/43 base pair bands must be of paternal origin implying that, of the two maternal alleles, she has inherited the one represented by the 142 base pair band and, since it has been establised that this allele is not associated with haemophilia in this family, it can be said that she is not a carrier of haemophilia.

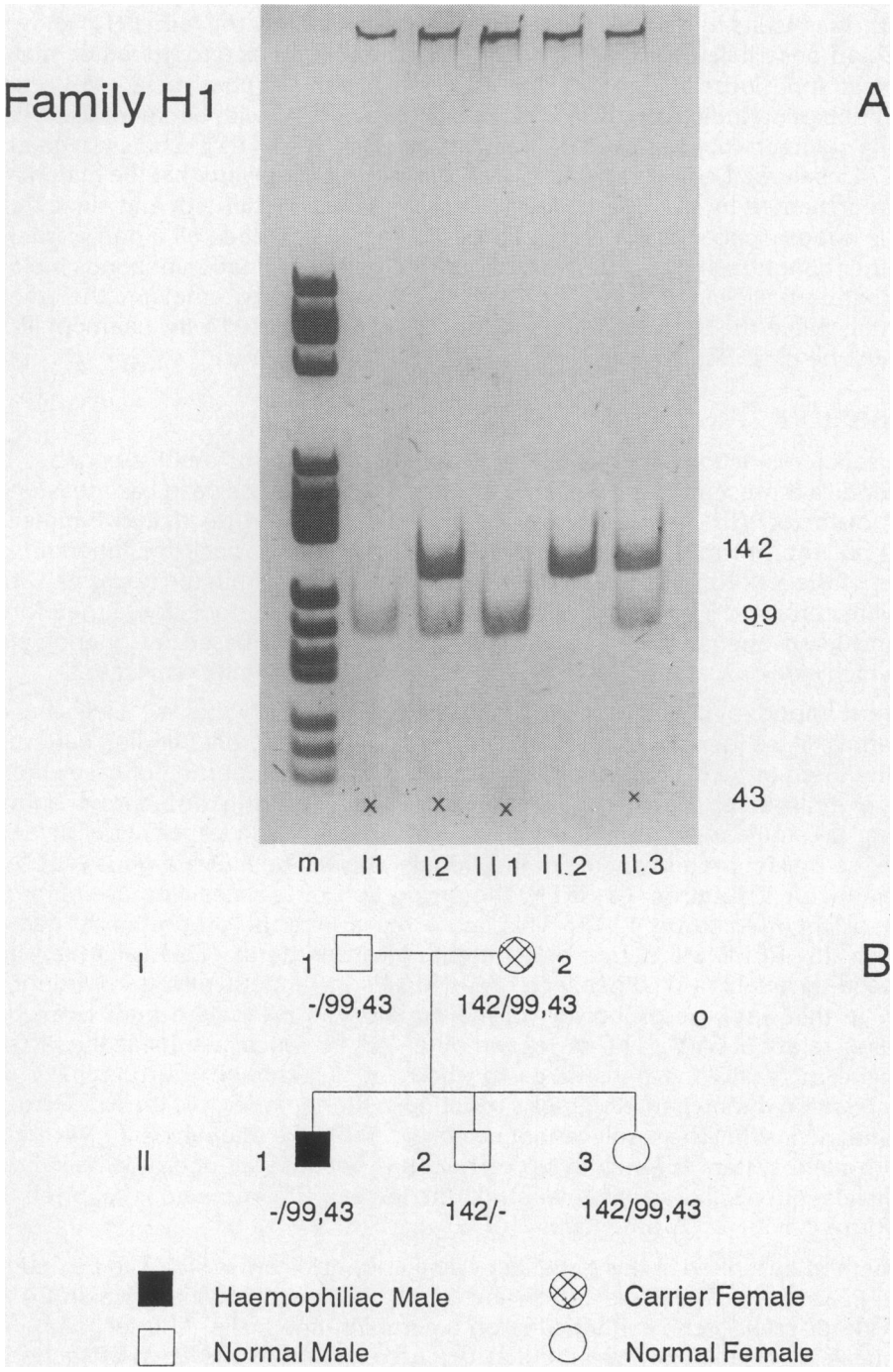

Fig 2. Family H1. (A) Polyacrylamide gel showing *Bcl1* digests of amplified DNA. (B) Family tree showing intron 18 genotypes and carrier status of individuals. The 43 base pair bands are only faintly visible and have been marked X.

Fig 3 shows results for family H2. As in the previous family, the father (I1) shows the 99/43 base pair combination and the mother (I2) is heterozygous demonstrating all three possible bands. The haemophilic son (II1) has the 99/43 base pair combination indicating the maternal allele associated with haemophilia. The patterns obtained for the two daughters are different. One (II2) is heterozygous; the 99/43 base pair combination are of paternal origin, implying that the maternal allele represented by the 142 base pair bands has been inherited, and since this allele is not associated with haemophilia, she can be classified as a non-carrier. The other daughter shows only the 99/43 base pair combination of bands indicating that both alleles possess a *Bcl1* site, one of which is paternal and the other maternal. Since the maternal allele in this case is associated with haemophilia, she must be classified as a carrier.

DISCUSSION

The use of restriction fragment length polymorphisms to identify carriers of haemophilia represents an improvement on previous methods based on measurements of factor VIII plasma clotting activity.[3] Because the restriction fragment length polymorphism used is intragenic, it was possible to track the inheritance pattern of the X chromosome carrying the mutated gene within the pedigree with complete certainty. The results of carrier testing by this method should therefore be considered unequivocal. This contrasts with methods based on phenotypic data which give only a probability of carriership and have a finite error rate.[1]

The most important limitation of this method is the necessity that the alleles have different DNA sequences at the RFLP, which generate different banding patterns allowing them to be distinguished from each other. In a proportion of cases both alleles of a carrier female will have the same sequence, making the X chromosome carrying the mutation indistinguishable from its homologous partner. For any RFLP, the maximum percentage of individuals who will be heterozygous is 50%. For the factor VIII intron 18 RFLP, the proportion of females who are heterozygous is reported to be 42%.[4] This figure represents the proportion of cases in which this RFLP will permit assignment of carrier status. This value can be increased by analysis of other factor VIII RFLPs in tandem with the intron 18 RFLP. In this way, the proportion of individuals who are heterozygous can be increased to about 65%. The use of two other RFLPs which lie outside the factor VIII gene increases the percentage to about 90% although the possibility of recombination during gametogenesis resulting in the separation of the RFLP from the gene means that the result cannot be considered to be unequivocal.[3] Recently a multi-allele system for identifying carriers has been developed. It is based on repetitive sequences of variable length in the factor VIII gene, and is reported to be informative for 95% of females.[11]

The method described in this paper allows haemophilia carrier status to be determined in about 40% of cases. It can be carried out rapidly (usually within 24h) and without great technical difficulty on a semi-routine basis. The technique is potentially applicable to any inherited disorder for which the aberrant gene is known and for which RFLPs have been characterised. The approach represents an important interface between clinical medicine and the rapidly expanding field of molecular biology.

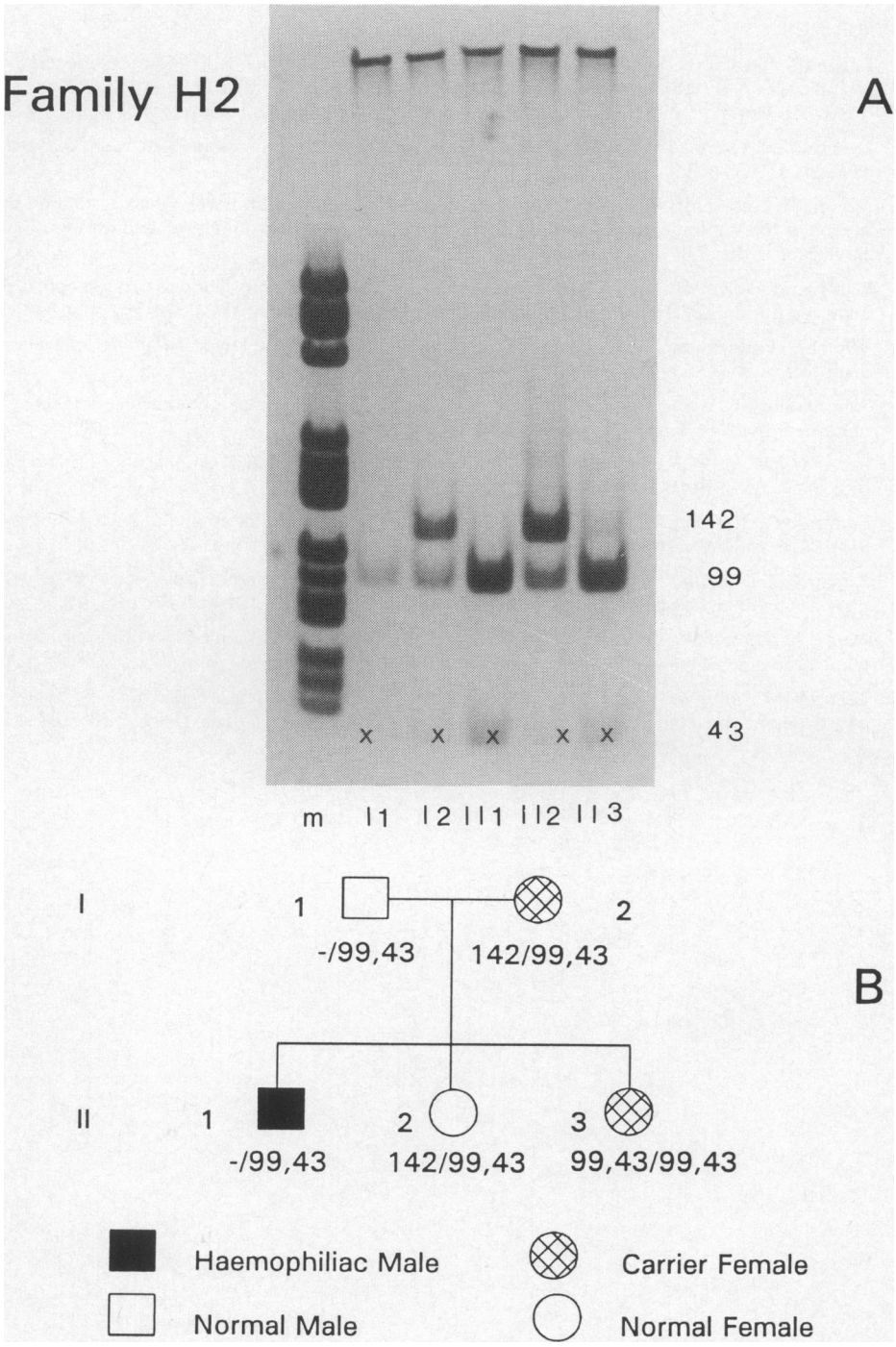

Fig 3. Family H2. (A) Polyacrylamide gel showing *Bcl1* digests of amplified DNA. (B) Family tree showing intron 18 genotypes and carrier status of individuals.

REFERENCES

1. Peake IR, Newcombe RG, Davies BL, Furlong RA, Ludlam CA, Bloom AL. Carrier detection in haemophilia A by immunological measurement of factor VIII related antigen (VIIIRAg) and factor VIII clotting antigen (VIIIAg). *Brit J Haematol* 1981; **48**: 651-60.
2. Lyon M. Sex chromatin and gene action in the mammalian X-chromosome. *Am J Hum Genet* 1962; **14**: 135-48.
3. Moodie P, Liddell MB, Peake IR, Bloom AL. Carrier detection in 50 haemophilia A kindred by means of three intragenic and two extragenic restriction fragment length polymorphisms. *Br J Haematol* 1988; **70**: 77-84.
4. Gitschier J, Drayna D, Tuddenham EGD, *et al.* Genetic mapping and diagnosis of haemophilia A achieved through *Bcl1* polymorphism in the factor VIII gene. *Nature* 1985; **314**: 738-40.
5. Wion KL, Tuddenham EGD, Lawn RM. A new polymorphism in the factor VIII gene for prenatal diagnosis of haemophilia A. *Nuc Acids Res* 1986; **14**: 4535-42.
6. Antonarakis SE, Waber PG, Kittur SD, *et al.* Haemophilia A: detection of molecular defects and of carriers by DNA analysis. *N Engl J Med* 1985; **313**: 842-8.
7. Kazazian HH. In: PCR technology: principles and applications for DNA amplification. Erlich HA (ed). New York: Stockton Press, 1989: 153-69.
8. Sambrook J, Fritsch EF, Maniatis T. In: Molecular cloning: a laboratory manual. 2nd ed. Nolan C (ed). New York: Cold Spring Harbor Laboratory Press, 1989: 9. 16-9. 19.
9. Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. *N Engl J Med* 1987; **317**: 985-90.
10. Harper K, Winter RM, Pembrey ME, Hartley D, Davies KE, Tuddenham EGD. A clinically useful DNA probe closely linked to haemophilia A. *Lancet* 1984; **ii**: 6-8.
11. Lalloz MRA, McVey JH, Pattinson JK, Tuddenham EGD. Haemophilia A diagnosis by analysis of a hypervariable dinucleotide repeat within the factor VIII gene. *Lancet* 1991; **338**: 207-11.